Rapid detection of *Mycoplasma pneumoniae* in clinical samples by the polymerase chain reaction

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Summary. A DNA amplification method was used to detect *Mycoplasma pneumoniae* in clinical samples. *M. pneumoniae* 16S ribosomal RNA gene sequences were selected as the amplification target region. The polymerase chain reaction (PCR) with purified DNA fragments as templates yielded an expected 88-bp fragment from *M. pneumoniae* but not from other *Mycoplasma* spp. nor from any of the other bacteria assayed. With this method, the 88-bp product specific for *M. pneumoniae* could be obtained from a minimum of 0.05 pg of *M. pneumoniae* DNA. Subsequently this PCR technique was used for the detection of *M. pneumoniae* in throat-swab samples. Twenty-two of 30 culture-positive clinical samples gave positive results in the PCR test. Thirty-two culture-negative clinical samples and 33 samples from healthy volunteers, of which only one was culture-positive, gave negative results in the same PCR test. This PCR method is useful for the direct detection of *M. pneumoniae* in clinical samples.

Introduction

Mycoplasma pneumoniae is one of the causative agents of primary atypical pneumonia, especially in children and young adults.^{1,2} Epidemic infection with *M. pneumoniae* tends to occur at intervals of 3–5 years, but most childhood cases are mild. To avoid the unnecessary use of ineffective antibiotics, a rapid method of diagnosis is needed.

Serological methods such as the complement fixation test (CFT)³ and indirect haemagglutination (IHA)⁴ are available for the diagnosis of *M. pneumoniae* infection, but these tests are time-consuming, have poor sensitivity and specificity, and diagnosis is often retrospective. Recently, enzyme-linked immunosorbent assays (ELISAs) with *M. pneumoniae* antigen or antibody have been developed⁵ and a rapid diagnostic method based upon DNA probe technology is also available.^{6–9} These are more specific and sensitive than the serological methods, but the minimum detectable number of cells is thought to be 10³–10⁵ cfu for the DNA probe method, and radioisotope detection is required.

The results of the DNA probe procedure suggested that the polymerase chain reaction (PCR) method might be useful for clinical diagnosis.¹⁰ This method amplifies a specific region of DNA by using two primers and Taq polymerase. Detection of *M. pneu-*

moniae DNA by PCR has been reported by several groups,^{11,12} but has not yet been applied to human clinical samples. We report here the detection of *M. pneumoniae* in clinical throat-swab specimens by a PCR procedure.

Materials and methods

Bacterial strains and growth conditions

M. pneumoniae strains FH and MAC, *M. buccale* NIH-CH-20247, *M. faucium* ATCC 25293A, *M. fermentans* fermentans-C, *M. genitalium* G37, *M. hominis* PG21, *M. orale* ATCC 15539 and *M. salivarium* ATCC 14277 were kindly supplied by Dr M. Yoshikawa, The Institute of Medical Science, The University of Tokyo. They were grown on modified Hayflick's medium supplemented with horse serum 10%,¹³ except for *M. genitalium* which was grown on medium supplemented with fetal bovine serum 10%. After incubation for 3–5 days at 37°C, they were subcultured in fresh medium. A diphasic medium^{14,15} was used for cultivation of clinical samples to isolate *M. pneumoniae*. The bacterial strains investigated are listed in table I.

Clinical samples

The throat-swab samples were obtained from paediatric outpatients presenting with symptoms of cough and fever at several hospitals in Shizuoka prefecture,

Table I. Bacterial species used for evaluation of the specificity of the PCR detection method

<i>Bacillus subtilis</i>
<i>Escherichia coli</i>
<i>Haemophilus influenzae</i>
<i>Klebsiella pneumoniae</i>
<i>Micrococcus</i> sp.
<i>Mycobacterium tuberculosis</i>
<i>Neisseria</i> sp.
<i>Proteus</i> sp.
<i>Pseudomonas aeruginosa</i>
<i>Serratia marcescens</i>
<i>Staphylococcus aureus</i>
<i>S. epidermidis</i>
<i>Streptococcus pneumoniae</i>
<i>S. pyogenes</i>
<i>S. salivarius</i>
<i>Actinomyces israelii</i>
<i>Bacteroides fragilis</i>
<i>B. ureolyticus</i>
<i>Eubacterium aerofaciens</i>
<i>Peptostreptococcus magnus</i>
<i>Mycoplasma buccale</i>
<i>M. faucium</i>
<i>M. fermentans</i>
<i>M. genitalium</i>
<i>M. hominis</i>
<i>M. orale</i>
<i>M. pneumoniae</i>
<i>M. primatum</i>
<i>M. salivarium</i>

Japan. Negative control samples were collected from healthy volunteers. Swabs were washed out in 1 ml of PPLO broth by vortex mixing. For detection of *M. pneumoniae* by cultivation, 0.1 ml of the suspension was inoculated into the diphasic medium and incubated at 37°C for 2–3 weeks. The remainder of the sample was used for DNA extraction.

Extraction of DNA

DNA was extracted as described by Stauffer *et al.*¹⁶ Briefly, 10 ml of broth-grown mycoplasmas were centrifuged at 15000 *g* for 10 min at 4°C and the supernate was discarded. The pellet was suspended in 100 µl of 0.15 M NaCl, 0.1 M EDTA solution, and then 400 µl of lysis solution (0.1 M NaCl; 0.1 M Tris-HCl, pH 8.0; SDS 1%) was added. The solution was then frozen at –80°C for 10 min, and incubated at 60°C for 5 min after thawing. The lysate was extracted gently, twice, with an equal volume of phenol:chloroform (1:1). DNA was incubated at 37°C for 2 h with RNAase A (Sigma) 50 µg/ml. The DNA was precipitated with two volumes of ice-cold absolute ethanol, rinsed twice with ethanol 70%, and after drying by evaporation, suspended in 100 µl of TE (10 mM Tris-HCl, pH 8.0; 1 mM EDTA). The concentration of DNA was measured with a spectrophotometer (Beckman Du-65, Beckman Instruments Inc., Fullerton, USA). For the extraction of bacterial DNA, cells grown in LB broth (Bacto-Trypton 1%, yeast extract 1%, NaCl 0.5%) were harvested by centrifugation at 10000 *g* for 1 min followed by the same procedure as

described above. For throat swabs, the PPLO broth (1 ml) rinse was centrifuged at 15000 rpm for 10 min at 4°C, and the pellet was suspended in 400 µl of 15 mM EDTA, 0.1 M NaCl, and vortex mixed. To this was added 50 µl of 0.1 M NaCl, 0.1 M Tris-HCl (pH 8.0), SDS 8%, and then the solution was frozen. Further extraction was performed as described above. Finally the samples were suspended in 10 µl of TE, and 2 µl of the suspension was subjected to the PCR assay.

DNA amplification

Oligonucleotide primers were synthesised by a DNA synthesiser (Model 380B, Applied Biosystems Inc., Foster City, USA). Amplifications were performed in a thermal cycler TSR-300 (Iwaki Glass Co., Tokyo, Japan) by the method described by Saiki *et al.*¹⁷ The PCR reaction mixture consisted of 50 µl of 200 µM dNTPs, 5 µl of 10 × Taq polymerase reaction buffer (Stratagene, La Jolla, USA) (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM MgCl₂; gelatin 0.1% w/v and other stabilisers), an appropriate amount (1–10 µl) of template DNA, 1 µM of each primer, 2.5 units of Taq polymerase (Stratagene), and water. To avoid evaporation, two drops of mineral oil were poured on top of the reaction mixture. The temperature programme was as follows: 94°C for 120 s for initial denaturation, 94°C for 60 s for cycle denaturation, 62°C for 30 s for annealing, 74°C for 30 s for extension. After 35 cycles of incubation, the mixture was incubated at 74°C for 60 s for additional extension. The 10 µl of PCR products were analysed by agarose 4% gel electrophoresis in Tris-borate EDTA buffer (Nusieve GTG: Agarose ME = 3:1) (FMC Bio-Products, Rockland, USA). The gel electrophoresis was performed at 100 V for 2.5–3 h. DNA fragments were visualised by UV illumination after staining with ethidium bromide 0.5 µg/ml for 30 min at room temperature.

Oligonucleotide primers for PCR

Oligonucleotide primers were selected from a variable region of the *M. pneumoniae* 16S rRNA genome¹⁸ as shown in fig. 1. The sense primer designated Mp-8 consisted of 22 oligonucleotides and the anti-sense primer designated Mp-6 consisted of 26 oligonucleotides. The extra primer, Mp-1, for three-primer

Fig. 1. Oligonucleotide primers on 16S rRNA gene of *M. pneumoniae* used for the detection of *M. pneumoniae* by PCR.

PCR, was a 26 oligonucleotide sense primer. These oligonucleotide sequences were 5'-CAAAGTTGAA-AGGACCTGCAAG-3' for Mp-8, 5'-ATTGCCTTG-GTAGGCCGTTACCCAC-3' for Mp-6, and 5'-ACACGTATCCAATCTACCTTATAATG-3' for Mp-1. The fragment amplified by Mp-8 and Mp-6 was 88 bp, and that amplified by Mp-1 and Mp-6 was 172 bp.

Three-primer PCR

To confirm that the amplified DNA originated from *M. pneumoniae*, three primer PCR was performed as described previously¹⁹ with Mp-8, Mp-6 and Mp-1. When PCR with these three primers produced simultaneous amplification products of 88 bp and 172 bp it could be assumed that these had originated from the target region.

Results

Specificity of PCR for *M. pneumoniae*

No DNA amplification was detected by this method when non-mycoplasma bacterial DNA was examined (data not shown). The results of PCR of eight *Mycoplasma* species are shown in fig. 2. The specific 88-bp amplification product was produced from *M. pneumoniae* strains MAC and FH (fig. 2, lanes 8 and 9) but not from other *Mycoplasma* spp. (fig. 2, lanes 2–7, 10 and 11). The three-primer method produced two specific bands, 88 and 172 bp, indicating that the 88-bp product of the amplification of Mp-8 and Mp-6 originated from *M. pneumoniae* (fig. 2, lane 12). A 50-bp fragment sometimes appeared in negative controls. This fragment seemed to be a primer dimer and affected neither the specificity nor the sensitivity of the PCR for *M. pneumoniae*.

Sensitivity of PCR for *M. pneumoniae*

The sensitivity of the PCR method was examined by

Fig. 2. Specificity of the PCR method for the detection of *M. pneumoniae*: 10 ng of DNA extracted from *Mycoplasma* spp. was used as a template in PCR. Lanes 1 and 14, marker DNA (*Hae*III-digested pAT153 DNA); 2, *M. buccale*; 3, *M. faucium*; 4, *M. fermentans*; 5, *M. genitalium*; 6, *M. hominis*; 7, *M. orale*; 8, *M. pneumoniae* FH strain; 9, *M. pneumoniae* MAC strain; 10, *M. primatum*; 11, *M. salicarium*; 12, *M. pneumoniae* FH strain (amplified by three-primers PCR); 13, no template.

serial dilutions of purified DNA from *M. pneumoniae* FH (fig. 3). PCR produced a visible 88-bp fragment from 0.05 pg of template DNA indicating that c. 100 organisms could be detected. When the amplified products were hybridised with a complementary probe, the sensitivity was raised c. 100-fold (data not shown).

Detection of *M. pneumoniae* in throat-swab samples by PCR

Seventy-two throat swabs were obtained from patients suspected of having *M. pneumoniae* infection. Thirty samples were culture-positive for *M. pneumoniae*, 32 were negative and 10 gave indeterminate results due to bacterial contamination. In the PCR tests, the 88-bp fragment was produced from 22 of 30 culture-positive samples, two of 32 culture-negative samples and three of 10 contaminated samples (table II). In contrast, none of the specimens from healthy controls produced the 88-bp band, although one sample was culture-positive. Representative PCR results are shown in fig. 4.

Discussion

A PCR procedure was developed for detection of *M. pneumoniae* in clinical throat swabs. Two PCR

Fig. 3. Sensitivity of PCR for detection of *M. pneumoniae*: various doses (0.01 pg–10 ng) of *M. pneumoniae* DNA (FH strain) were used as templates in the PCR. Lanes 1 and 11, marker DNA (*Hae*III-digested pAT153 DNA); 2, 10 ng; 3, 1 ng; 4, 100 pg; 5, 10 pg; 6, 1 pg; 7, 0.1 pg; 8, 0.05 pg; 9, 0.01 pg; 10, no template.

Table II. Evaluation of PCR and cultivation methods for detection of *M. pneumoniae* in throat swabs from patients and healthy volunteers

Throat swab source	Culture result	Number of samples tested	Number of samples positive in PCR
Patient	+	30	22
	–	32	2
	C*	10	3
Healthy volunteer	+	1	0
	–	32	0

* Culture result was not reliable due to contamination with other bacteria.

Fig. 4. Detection of *M. pneumoniae* by PCR in throat swabs obtained from patients suspected to have *M. pneumoniae* infection and from healthy volunteers. Lanes 1 and 13, marker DNA (*Hae*III-digested pAT153 DNA); 2–4, throat swabs from patients (culture-positive); 5–7, throat swabs from patients (culture-negative); 8–10, throat swabs from healthy volunteers (culture-negative); 11, *M. pneumoniae* (FH strain) DNA, 10 ng; 12, no template.

primers, Mp-6 and Mp-8, were selected from a variable region of the 16S rRNA gene which has specific sequences in many bacterial strains.^{18,20,21} The target 88-bp fragment was amplified in tests with *M. pneumoniae* FH and MAC but not with other *Mycoplasma* spp. such as *M. buccale*, *M. faucium*, *M. fermentans*, *M. genitalium*, *M. hominis*, *M. orale*, *M. primatum* and *M. salivarium*, as reported previously.^{9–12} The 88-bp fragment was not amplified in tests with other bacteria. Three-primer PCR was also performed with an additional primer Mp-1. The production of two amplified products of 88 and 172 bp indicated that the 88-bp product originated from *M. pneumoniae* DNA.

The PCR method amplified the 88-bp fragment from 0.05 pg DNA of *M. pneumoniae*, indicating that the PCR can theoretically detect 100 *M. pneumoniae* cells. The high sensitivity and specificity of mycoplasma PCR demonstrated in this study were similar

to results reported by others.^{11,12} Bernet *et al.*¹¹ reported that PCR can detect 10^2 – 10^3 organisms, and Jensen *et al.*¹² estimated a detection limit of < 40 cfu.

We applied this PCR method to the detection of *M. pneumoniae* in clinical specimens. The 88-bp band was amplified with 22 of 30 culture-positive throat samples. It is unclear why eight culture-positive samples did not give a positive PCR result. This may have been due to poor recovery of DNA during the extraction procedure. Two of 32 culture-negative clinical samples gave a positive reaction in PCR, indicating a relatively high specificity of PCR in comparison with cultivation techniques. It is possible that our cultivation technique with a diphasic medium^{14,15} was not specific for the detection of *M. pneumoniae*. The cultivation method depends on a colour change due to acid produced by growth of *M. pneumoniae*, but the growth of contaminating bacteria can also change the colour of the medium. No positive PCR result was obtained with 33 samples from healthy volunteers.

Jensen *et al.*¹² used PCR to detect mycoplasmas in simulated clinical samples. Similarly, Bernet *et al.*¹¹ used the PCR method for biological samples (throat swabs) obtained from hamsters experimentally infected with *M. pneumoniae*. The present study is the first to use PCR for the detection of *M. pneumoniae* in human clinical samples (throat swabs). The sensitivity and specificity of PCR were estimated to be 73% and 94%, respectively. Further evaluation of this PCR method is needed if the test is to be used routinely in clinical practice.

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